UNIVERSITI PUTRA MALAYSIA

SEMISYNTHESIS OF ANDROGRAPHOLIDE DERIVATIVES AND EVALUATION OF THEIR ANTITUMOUR PROPERTIES

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By

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Chairman: Associate Professor Nasaruddin bin Abdul Aziz, M.D. M.Med. Sc.

Faculty: Medicine and Health Sciences

Previously, andrographolide, which is the major diterpenoid of *Andrographis paniculata*, was shown to have *in vivo* antitumour activity against human breast tumour xenografts. In this study, among the four compounds isolated from *A. paniculata*, andrographolide was the most potent compound with a mean IC_{50} value of 8 μM in MCF-7 human breast cancer cells. Neoandrographolide showed a weak cytotoxic effect, whereas 14-deoxy-11,12-didehydroandrographolide and 14-deoxyandrographolide failed to exhibit growth inhibitory effect at the highest tested concentration of 100 μM. Owing to this, andrographolide was considered as the lead compound in the discovery of potent and selective antitumour agents.

Using andrographolide isolated from *A. paniculata* as one of the starting materials, 3,19-

benzylidene andrographolide and 3,19-alkylidene andrographolide derivatives were synthesised by coupling of the two -OH groups present at C-3 and C-19 of andrographolide with different benzaldehydes and alkyl aldehydes, respectively. In addition, new derivatives were also synthesised by acetylation, oxidation, Heck and esterolysis reactions. The structures of new derivatives of andrographolide derivatives were confirmed by spectral analysis ($^{1}H/^{13}C$ NMR, MS, FT-IR, UV).

Forty seven compounds including andrographolide were tested for antitumour activities in MCF-7 and HCT-116 (colon) cancer cell lines. Using a 72 h MTT cell viability assay, parameters of dose-response effects, GI_{50}, TGI and LC_{50} were determined. The derivatives had submicromolar GI_{50} values, except for 3,19-(4-nitrobenzylidene)andrographolide (**SRJ58**), which showed the most potent activity with a GI_{50} value of 0.7 μM in MCF-7 cells. Only (Z)-2-[1-benzylamino-2-(5,5,6,8a-tetramethyl-2-methylene-decahydro-naphthalen-1-yl)-ethyl]-4-hydroxy-but-2-enoic acid benzylamide] (**SRJ18**), displayed a pronounced selectivity (approximately 8-fold) towards HCT-116 cells at the GI_{50} value compared with MCF-7 cells.

Out of the five compounds (3,19-isopropylideneandrographolide (**SRJ01**), 14-acetylandrographolide (**SRJ03**), 3,19-(2-bromobenzylidene)-14-deoxy-11,12-didehydro andrographolide (**SRJ05**), 3,19-(2-bromobenzylidene)andrographolide (**SRJ09**) and 3,19-(3,4-dimethoxybenzylidene)andrographolide (**SRJ13**)) tested against the 60 National Cancer Institute (NCI) of USA human cancer cell lines, only **SRJ09** showed some form of selectivity towards cancers of the colon, central nervous system, renal and melanoma. The mechanism(s) of actions of the compounds were also studied by

determining their effect in inducing cell cycle arrest and apoptosis. Andrographolide, **SRJ01** and **SRJ03** induced G_1 and G_2/M arrest in MCF-7 cells, whereas 3,19-(4-bromobenzylidene)andrographolide (**SRJ08**), **SRJ09**, 3,19-(3-bromobenzylidene) andrographolide (**SRJ10**), 3,19-(3-chloro-4-fluorobenzylidene)andrographolide (**SRJ23**) and 3,19-(2-fluorobenzylidene)andrographolide (**SRJ27**) induced only G_1-phase arrest in MCF-7 cells. **SRJ09** down-regulated CDK4 (a G_1-phase regulator) protein levels in MCF-7 cells, which explains the G_1-phase arrest by the compound. NCI's COMPARE mechanistic analysis revealed that the compounds antitumour activities were not similar to that of standard anticancer drugs with known mechanisms of action. Projection of **SRJ03** in the Self-Organising Maps (SOMs) analyses of NCI suggested that this compound may be targeting cell cycle related phosphatases or kinases. However, andrographolide, **SRJ01**, **SRJ05**, **SRJ09** and **SRJ13** did not project in the known mechanism categories.

The mode(s) of cell death induced by **SRJ09** and **SRJ23**, identified by fluorescence microscopy and flow cytometry, was confirmed to be apoptosis in HCT-116 cells.

In conclusion, novel derivatives of andrographolide, especially **SRJ09**, **SRJ18** and **SRJ58** are potential lead molecules for future antitumour studies to discover prospective clinical candidates.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai syarat memenuhi keperluan untuk Ijazah Doktor Falsafah

SEMISINTESIS TERBITAN ANDROGRAPHOLIDE DAN PENILAIAN CIRI-CIRI ANTITUMORNYA

Oleh

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Andrographolide merupakan diterpenoid utama tumbuhan *Andrographis paniculata* dan kajian terdahulu menunjukkan andrographolide mempunyai aktiviti anti-tumor secara *in vivo* terhadap xenograf tumor payudara manusia. Dalam kajian ini, andrographolide merupakan sebatian yang paling poten diantara empat sebatian daripada *A. paniculata*, dengan nilai min IC_{50} 8 μM dalam sel kanser payudara manusia MCF-7. Neoandrographolide mempamerkan kesan sitotoksik yang lemah, manakala 14-deoxy-11,12-didehydroandrographolide dan 14-deoxyandrographolide gagal menunjukkan kesan perencatan tumbesaran apabila diuji pada kepekatan tertinggi iaitu 100 μM. Justeru itu, andrographolide telah dipilih sebagai sebatian asas dalam usaha menghasilkan agen antitumor yang poten dan selektif berasaskan struktur rangka andrographolide.

Dengan menggunakan andrographolide yang diasingkan daripada *A. paniculata* sebagai bahan asas, 3,19-benzilidene andrographolide dan 3,19-alkilidene andrographolide disintesis dengan mengkupelkan dua kumpulan –OH pada kedudukan C-3 dan C-19 andrographolide masing-masing dengan benzaldehid dan alkil aldehid. Selain itu, terbitan andrographolide juga disintesis melalui proses asetilasi, oksidasi, tindakbalas Heck dan esterolisis. Struktur bagi terbitan baru andrographolide disahkan dengan menggunakan analisis spektral ($^{1}H/^{13}C$ NMR, MS, FT-IR, UV).

Kesemua sebatian termasuk andrographolide diuji untuk menentukan antitumor terhadap kultur kanser payudara, MCF-7 dan kanser kolon, HCT-116. Dengan menggunakan asai viabiliti sel MTT selama 72 jam, nilai GI_{50}, TGI dan LC_{50} ditentukan. Kesemua sebatian terbitan menunjukkan nilai GI_{50} submikromolar terhadap kedua-dua jenis sel terutamanya 3,19-(4-nitrobenzylidene)andrographolide (**SRJ58**), yang menunjukkan aktiviti paling poten dengan nilai GI_{50} pada 0.7 μM. Antara sebatian-sebatian tersebut, 8-kali ganda (Z)-2-[1-Benzylamino-2-(5,5,6,8a-tetramethyl-2-methylene-decahydro-naphthalen-1-yl)-ethyl]-4-hydroxy-but-2-enoic acid benzylamide (**SRJ18**), menunjukkan selektiviti terhadap sel HCT-116 dengan katara pada nilai GI_{50} berbanding sel MCF-7.

Daripada lima sebatian (3,19-isopropylideneandrographolide (**SRJ01**), 14-acetylandrographolide (**SRJ03**), 3,19-(2-bromobenzylidene)-14-deoxy-11,12-didehydro andrographolide (**SRJ05**), 3,19-(2-bromobenzylidene)andrographolide (**SRJ09**) and 3,19-(3,4-dimethoxybenzylidene)andrographolide (**SRJ13**)) yang telah diuji ke atas 60 jenis sel kanser oleh National Cancer Institute (NCI), USA, hanya **SRJ09** menunjukkan

selektiviti terhadap kanser sistem saraf pusat dan melanoma.

Andrographolide, **SRJ01** dan **SRJ03** didapati mengaruh perencatan fasa G_1 dan G_2/M pada sel MCF-7, manakala 3,19-(4-bromobenzylidene)andrographolide (**SRJ08**), **SRJ09**, 3,19-(3-bromobenzylidene) andrographolide (**SRJ10**), 3,19-(3-chloro-4-fluorobenzylidene)andrographolide (**SRJ23**) and 3,19-(2-fluorobenzylidene)-andrographolide (**SRJ27**) hanya merencatkan fasa G_1 pada sel MCF-7. Kesan **SRJ09** terhadap (oerangsangan hitaran regulaton cyclin) yang bergantung terhadap kinase 4 (CDK4) telah ditentukan melalui analisis Western blot. **SRJ09** merencatkan tahap CDK4 pada sel MCF-7 setelah dirawat selama 72 jam. Analisis NCI COMPARE menunjukkan mekanisme aktiviti sebatian-sebatian ini, tidak sama seperti yang ada pada dadah antikanser yang diketahui. Projeksi **SRJ03** dalam analisis 'Self-Organising Maps' (SOMs) mencadangkan mekanisma tindakannya berkemungkinan bersasar ke atas enzim fosfatase atau kinase. Walau bagaimanapun, andrographolide, **SRJ01**, **SRJ05**, **SRJ09** dan **SRJ13** tidak dipamerkan dalam kategori mekanisma yang diketahui.

Mekanisma kematian sel yang diaruh oleh agen baru ini dikenalpasti melalui pemerhatian mikroskop pendaflor dan 'sitometri aliran'. Daripada kedua-dua kaedah ini, apoptosis dikenal pasti sebagai mekanisma utama kematian sel HCT-116 yang dirawat dengan **SRJ09** dan **SRJ23**.

Secara kesimpulan, sebahagian sebatian terbitan andrographolide, terutamanya **SRJ09**, **SRJ18** dan **SRJ58** menpunyai potensi sebagai komponen utama kajian antitumor untuk menemui calon klinikal yang bekesan di masa hadapan.

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I certify that an Examination Committee met on 21st October 2004 to conduct the final examination of Jada Srinivasa Rao on his Doctor of Philosophy thesis entitled "Semisynthesis of Andrographolide Derivatives and Evaluation of Their Antitumour Properties" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements for the degree of Doctor of Philosophy. The members of the supervisor committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

JSRao

JADA SRINIVASA RAO

Date: 06/12/2004

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ab-1	Actin
AO	acridine orange
AMPS	ammonium persulfate
AG	andrographolide
ATP	adenosine triphosphate
BSA	bovine serum albumin
CDK	cyclin-dependent kinase
CDKI	cyclin-dependent kinase inhibitor
CNS	central nervous system
COMPARE	Computerised Pattern-recognition algorithm
DAPI	4,6-diamino-2-phenyl indole
DMSO	dimethyl sulfoxide
DMF	dimethyl formamide
DNA	deoxyribonucleic-acid
ECL	enzyme chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EGTR	ethylene glycol-bis (β-aminoethyl ether) *N, N, N', N'*-tetraacetic acid
FACS	fluorescence-activated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GI_{50}	50% growth inhibition

H_2O	distilled water/sterile water
HPLC	high-pressure liquid chromatography
HRP	horseradish peroxidase
IC_{50}	50% inhibition concentration
LC_{50}	50% lethal concentration
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NCI	National Cancer Institute
PBS	phosphate-buffered saline
PCC	Pearson correlation coefficient
PI	propidium iodide
PS	phosphatidylserine
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
RNase	ribonuclease
RPMI	Roswell Park Memorial Institute
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS–PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOM	self-organising maps
TCM	traditional Chinese medicine
TEMED	*N,N,N',N'*-tetramethylethylenediamine
TGI	total growth inhibition
THF	tetrahydrofuran
TLC	thin layer chromatography

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

The use of plants as medicines goes back to early man. Certainly the great civilisations of the ancient Indians, Chinese, and North Africans provided written evidence of man's ingenuity in utilising plants for the treatment of a wide variety of diseases. In ancient Greece, scholars classified plants and gave descriptions of them thus aiding the identification process. It was not until the 19th century that man began to isolate the active principles of medicinal plants and one particular landmark was the discovery of quinine from *Cinchona* bark by the French scientists Caventou and Pelletier. Such discoveries led to an interest in plants from the New World and expeditions scoured the almost impenetrable jungles and forests in the quest for new medicines (reviewed by Phillipson, 2001). Despite major scientific and technological progress in combinatorial chemistry, drugs derived from natural products still make an enormous contribution to drug discovery today (reviewed by Phillipson, 2001).

Nature is an attractive source of new therapeutic candidate compounds and has a tremendous chemical diversity found in millions of species of plants, animals, marine organisms and microorganisms. The development of novel agents from natural sources presents obstacles that are not usually met when one deals with synthetic compounds. For instance, there may be difficulties in accessing the source of the samples, obtaining appropriate amounts of the sample, identification and isolation of the active compound in the sample, and problems in synthesising the necessary amounts of the compound of interest (Rocha *et al.*, 2001).

There are about 500,000 species of plants growing on the earth and it is estimated that at least 5000 different chemical compounds of secondary metabolites are present in a single species of plant (reviewed by Verpoorte, 1998). It is apparent that the secondary metabolites of plant origin constitute a tremendous resource for exploring useful drugs. In plants, the primary metabolites, including proteins, lipids, nucleic acids, enzymes, and coenzymes, etc., come from the metabolism of carbohydrates with the incorporation of nitrogen and mineral elements. By utilising primary metabolites and numerous infinite molecules, plants synthesise the secondary metabolites for the purpose of survival and well being. Taxonomically related plants generally produce chemically similar secondary metabolites and, therefore, may have similar pharmacological effects. Natural products exhibiting antitumour activity continue to be the subject of extensive research aimed at the development of drugs for the treatment of different human tumours.

In the early 1950s, a research program screening for antitumour drugs of plant origin was initiated mainly by the National Cancer Institute (NCI) in the USA. Large-scale screening procedures were made available, plant materials were produced, and crude extracts were put through preliminary screening. Basic pharmacological and toxicological studies in animals ensued, and finally, a number of promising compounds were selected for chemical studies, with the ultimate goal of finding the active antitumour drugs from plants. This program represented a combined effort mobilising many biomedical research organisations in the government and in medical, pharmaceutical, and chemical institutes and industries. The achievements during the past few decades have been very rewarding (reviewed by Cragg *et al.*, 1999).